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Culturing Selects for Specific Genotypes of *Borrelia burgdorferi* in an Enzootic Cycle in Colorado

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In Colorado, *Borrelia burgdorferi* sensu stricto, the etiologic agent of Lyme disease, is maintained in an enzootic cycle between *Ixodes spinipalpis* ticks and *Neotoma mexicana* rats (27). The frequencies of flagellin (*fla*), 66-kDa protein (*p66*), and outer surface protein A (*ospA*) alleles were examined in 71 *B. burgdorferi* isolates from samples from Colorado. Approximately two-thirds of these samples were isolates from *I. spinipalpis* ticks that had been cultured in BSK-H medium prior to DNA extraction. The remaining samples were from total DNA extracted directly from infected *I. spinipalpis* ticks. A portion of each gene was amplified by PCR and screened for genetic variability by single-strand conformation polymorphism (SSCP) analysis. We identified three alleles in the *fla* gene, seven in the *p66* gene, and seven in the *ospA* gene. Sequencing verified that the amplified products originated from *B. burgdorferi* template DNA and indicated 100% sensitivity and specificity of the SSCP analysis. The frequencies of the *p66* and *ospA* alleles were significantly different between cultured and uncultured spirochetes. The number of three-locus genotypes and the genetic diversity of alleles at all loci were consistently lower in cultured spirochetes, suggesting that culturing of *B. burgdorferi* in BSK-H medium may select for specific genotypes.

Borrelia burgdorferi Johnson, Schmid, Hyde, Steigerwalt & Brenner sensu lato is the etiologic agent of Lyme disease and related disorders worldwide (8, 11). *B. burgdorferi* sensu lato is maintained in enzootic cycles involving ticks of the genus *Ixodes* and rodent or lagomorph reservoirs (9, 27, 32, 43). In the foothills of Colorado, where no known indigenous cases of Lyme disease occur, *B. burgdorferi* is maintained in an enzootic cycle by *Ixodes spinipalpis* and the Mexican wood rat (*Neotoma mexicana*) (27). Other vertebrate hosts may also be involved because *I. spinipalpis* has been found on 22 mammal species (including humans) and 3 species of birds (for a review, see reference 30). Due to the xeric conditions in Colorado, *I. spinipalpis* ticks are restricted to a nidicolous existence, making zoonotic transmission of *B. burgdorferi* improbable (12).

The *B. burgdorferi* sensu lato species complex consists of four genospecies and many genetically variable isolates that have not been fully characterized (1, 22–24, 28, 35). Three genospecies, *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, are distributed throughout Europe and Eurasia. The fourth genospecies, *Borrelia japonica*, is restricted to Asia (24). Many studies have described greater genetic diversity among European *B. burgdorferi* sensu lato isolates than among *B. burgdorferi* sensu stricto isolates from North America (2, 8, 26, 47, 48). However, genetic diversity among California *B. burgdorferi* isolates from rodents was similar to that found among European isolates (50, 51), and isolates from ticks in California also had high levels of molecular and protein variability (26, 43). Genetic variability in *B. burgdorferi* sensu lato is known to arise from point substitutions, insertions, deletions, plasmid rearrangements, loss of genes, and changes in protein expression (14, 19, 40, 41).

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In the present study, the amount of genetic heterogeneity among Colorado *B. burgdorferi* isolates was examined in portions of the genes encoding flagellin (*fla*) (21), a 66-kDa protein (*p66*) (38), and outer surface protein A (*ospA*) (15). Each gene was amplified by PCR and was screened for genetic variation by single-strand conformation polymorphism (SSCP) analysis (33). Novel gene sequences identified by SSCP analysis were then sequenced and used in phylogenetic analysis of the samples. *B. burgdorferi* DNA was collected in two ways. The first method is commonly used in sampling *B. burgdorferi* from field collections. Tick midguts were dissected, spirochetes were cultured in BSK-H medium, and DNA was extracted from the cultured isolate. In the second method, spirochete DNA was extracted directly from infected ticks. This approach allowed us to compare the number and frequencies of alleles in *B. burgdorferi* DNA taken directly from ticks with DNA obtained from spirochetes subjected to the isolation procedures with BSK-H medium.

MATERIALS AND METHODS

Material and DNA isolation. For 47 of the 71 samples, *B. burgdorferi* was cultured from ticks that had been removed from 88 *N. mexicana* rats sampled from the field (27). Ticks were externally disinfected, homogenized in BSK-H culture medium (Sigma Chemical Co., St. Louis, Mo.), incubated at 34°C, and examined weekly for 4 weeks by dark-field microscopy for spirochetes (27). Primary cultures were passed to fresh medium and allowed to grow for 6 to 8 days. The resulting passage 1 cultures were mixed with glycerol (final concentration, 30%) and were frozen in 1-ml portions at −70°C. For the 24 remaining samples, *I. spinipalpis* ticks were stored in 70% ethanol and DNA was isolated by a hexadecyltrimethylammonium bromide isolation procedure (6).

PCR. All reactions were completed in 50-μl volumes of reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 μM deoxynucleoside triphosphates, and 1 μM [each] primer). Reagents were pipetted into 500-μl microcentrifuge tubes and overlaid with approximately 25 μl of mineral oil. Tubes, oil, and buffer were exposed to UV light (260 nm) for 10 min at a distance of 10 cm to destroy potentially contaminating template DNA. *Borrelia* template DNA was then added through the oil. Reaction tubes were placed in a PTC-100 thermal cycler (MJ Research, Watertown, Mass.) and were heated at 95°C for 1 min; the temperature was reduced to 80°C, and 1 U of *Taq* DNA polymerase (Promega) was added to each reaction mixture. Amplification conditions are described below. A single negative control without template was run simultaneously with each set of PCRs and was processed at the

TABLE 1. Primers used for PCR and sequencing of *B. burgdorferi*[a]

Gene, location, and direction	Sequence
fla	
Outer	
Forward	5′-AAG TAG AAA AGT CTT AGT AAG AAT GAA GGA-3′
Reverse	5′-AAT TGC ATA CTC AGT ACT ATT CTT TAT AGA T-3′
Inner	
Forward	5′-CAC ATA TTC AGA TGC AGA CAG AGG TTC TA-3′
Reverse	5′-GAA GGT GCT GTA GCA GGT GCT GGC TGT-3′
p66	
Outer (*a* set)	
Forward	5′-CGA AGA TAC TAA ATC TGT-3′
Reverse	5′-GAT CAA ATA TTT CAG CTT-3′
Inner (*f* set)	
Forward	5′-TGC AGA AAC ACC TTT TGA AT-3′
Reverse	5′-AAT CAG TTC CCA TTT GCA-3′
ospA	
External	
Forward	5′-AAA AAA TAT TTA TTG GGA ATA GG-3′
Reverse	5′-GTT TTT TTG CTG TTT ACA CTA ATT GTT AA-3′
Internal	
Forward	5′-GGA GTA CTT GAA GGC G-3′
Reverse	5′-GCT TAA AGT AAC AGT TCC-3′

[a] The sequences of *fla* (21), *p66* (38), and *ospA* (15) were described previously.

end of the reaction set to detect any contaminants carried on plastics or pipettes. Entire reaction sets were discarded if amplified products appeared in the control.

Extraneous amplification products appeared frequently when using whole tick DNA as a template in the PCR and necessitated the use of nested PCR for the amplification of each gene. The nested protocol used 1 μl of the primary PCR product diluted 1:200 with water, and 2 μl of the diluted product was used as template DNA for the secondary PCR. Occasionally, a sample would require greater dilution of the primary product.

Amplification of the chromosomal flagellin (*fla*) fragment was completed with outer and inner primer sets (21) (Table 1). Amplification of the *B. burgdorferi*-specific DNA fragment was completed by using 50 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for the primary PCR. The secondary, internal PCR proceeded at 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min and used secondary, inner primers (Table 1).

B. burgdorferi-specific primers that amplify a "G2" region have been described (38) (Table 1). Later, this was identified as the chromosomal region encoding the 66-kDa protein (*p66*) (10, 36). Primer set *a* was used for primary PCR and involved 30 cycles of 94°C for 1 min, 37°C for 30 s, and 60°C for 1 min. The secondary PCR used the same program for an additional 25 cycles after adding primer set *f*.

The primary amplification of the plasmid-encoded *ospA* gene fragment was run for 20 cycles of 94°C for 30 s, 37°C for 30 s, and 72°C for 2 min (15) (Table 1). The secondary, internal amplification proceeded for 10 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and finally, 10 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min.

SSCP analysis. SSCP analysis (33) was completed with nondenaturing polyacrylamide gels formed with 0.6× TBE (1× TBE is 53 mM Trizma base [Sigma], 53 mM boric acid, and 1.5 mM EDTA [pH 8.0]), 7.9% acrylamide, and 0.21% *N,N′*-methylenebisacrylamide. From each PCR, 1 μl of product was removed to a 500-μl tube containing 9 μl of denaturing loading mix (20 mM NaOH, 90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). The tube was tapped to mix the contents, spun down, heated to 95°C for 2 min, and plunged into ice. From this cooled mixture, 2 μl was loaded onto a vertical SSCP analysis gel (16 by 18 cm; SE600 series; Hoefer Scientific Instruments, San Francisco, Calif.) that was run in refrigerated (4 to 6°C) circulating 0.5× TBE buffer. Amperage was maintained at 20 mA for approximately 2.5 h or until the xylene cyanol dye had migrated to the bottom of the gel. After electrophoresis, the gels were silver stained, dried, and photographed (4, 18, 29).

Genes for which all renatured single-strand and denatured single-strand bands had equal mobilities were assumed to have identical sequences and are hereafter referred to as an allele. Unique alleles from previous gels were included on all subsequent gels as references. In our experience, even slight shifts in renatured or denatured single-strand mobility are indicative of changes in the primary sequence (7, 13, 18, 29). When possible, two representatives of each unique allele were selected for sequencing to ensure accurate identification of alleles and to test the sensitivity and specificity of SSCP analysis.

DNA sequencing. Direct cycle sequencing was completed with amplified DNA from all samples (5). The PCR primers listed in Table 1 were used as primers to sequence both strands of the amplified product.

Sequence alignments and phylogenetic analysis. Sequences were manually read from autoradiographs by using SeqAid II, version 3.6 (37), and initially machine aligned by using CLUSTALV (17). The sequences were then manually aligned on codons, translated, and compared to published amino acid sequences of all three genes to ensure proper alignment.

Phylogenetic analyses were performed for each gene fragment separately. Maximum parsimony (MP), distance-neighbor joining (NJ), and maximum likelihood (ML) methods were used in all phylogeny reconstructions. PAUP, version 4.0 (45), was used to derive a ML tree (16). MP analyses were performed by using PAUP, version 4.0, and a bootstrap analysis with 100 replications was performed to test support for the *B. burgdorferi* sensu stricto clade. Genetic distances among taxa were estimated (46) and distance trees were derived by NJ analysis (39) with 100 bootstrap replications.

Allele frequencies were compared between cultured and uncultured (amplified from whole DNA extracted from an infected tick) *B. burgdorferi* strains by Fisher's exact test (44). Shannon's diversity index (*H*) was estimated as a general indicator of the amount of genetic diversity in a sample, where *H* is equal to $-\Sigma p_i \log_2 p_i$ and p_i is the frequency of allele i in a collection.

RESULTS

Three lines of evidence indicate that genes amplified from *I. spinipalpis* and the cultured isolates from Colorado are *B. burgdorferi* sensu stricto. First, the cultured isolates were identified as *B. burgdorferi* sensu lato by immunoblotting with monoclonal antibodies for OspA, OspC, and flagellin (27). Second, the *fla* primers used in this study are diagnostic for *B. burgdorferi* sensu lato (21). Third, phylogenetic analysis was performed on each gene by using sequences from JD1 and another *B. burgdorferi* sensu stricto isolate as in-groups and using the genes from *B. garinii*, *B. afzelii*, and *B. japonica* as out-groups (Fig. 1). In each case, the genes in this study formed

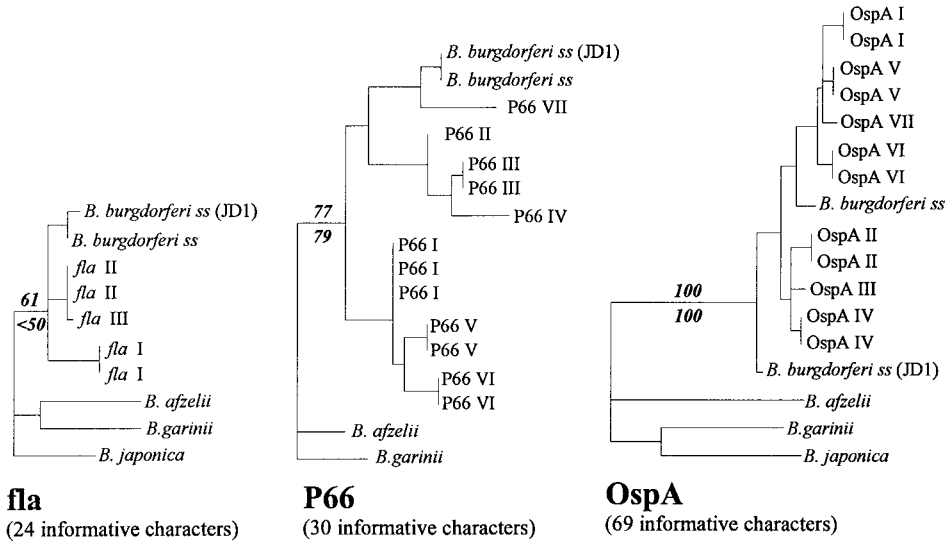

FIG. 1. ML trees (16) derived by using PAUP, version 4.0 (45). *B. burgdorferi* isolates from Colorado are indicated by the name of the gene and a roman numeral indicating the allele sequenced. GenBank accession numbers for Colorado *B. burgdorferi* isolates are U96234 to U96239 for *fla* and U96240 to U96252 for *p66* and *Borrelia burgdorferi* sensu stricto isolate JD1, and other *B. burgdorferi* sensu stricto isolates (*fla*, GenBank accession no. X69611; *p66*, GenBank accession no. X87725; *ospA*, GenBank accession no. S88693) were included in the in-group. *B. afzelii* (*fla*, GenBank accession no. X63413; *p66*, GenBank accession no. X87726; *ospA*, GenBank accession no. X65599), *B. garinii* (*fla*, GenBank accession no. X69598; *p66*, GenBank accession no. X87727; *ospA*, GenBank accession no. X65600), and *B. japonica* (*fla*, GenBank accession no. L29239; *ospA*, GenBank accession no. D29660) were used as out-groups. Branch lengths are proportional to percent divergence. NJ and parsimony analyses were performed with 100 bootstrap replications with PAUP, version 4.0. The frequency with which the *B. burgdorferi* sensu stricto clade was supported by MP analysis appears above the branch. The frequency with which the *B. burgdorferi* sensu stricto clade was supported by using distance (46) and NJ (39) analyses appears below the branch. *ss*, *B. burgdorferi* sensu stricto.

a monophyletic group with the genes from JD1 and another *B. burgdorferi* sensu stricto isolate. There was strong bootstrap support for monophyly in the *ospA* data set which contained 69 informative characters by the parsimony method. The *fla* and *p66* data sets also supported this relationship, but support by bootstrap analysis was lower because these data sets contained only 24 to 30 informative characters.

Three unique alleles were detected through SSCP analysis at the *fla* locus (Fig. 2), and seven unique alleles were detected at both the *p66* and *ospA* loci (Fig. 3A and B). Figures 2 and 3 demonstrate the reproducibility of the SSCP profile of an allele. Genes with identical SSCP patterns were sequenced to further test the specificity of the SSCP analysis. Duplicate alleles could not be analyzed in all cases because some alleles were detected only once. The numbers of transitions and transversions between pairs of alleles are listed in Table 2. In *fla*, two of the three alleles were sequenced from different isolates,

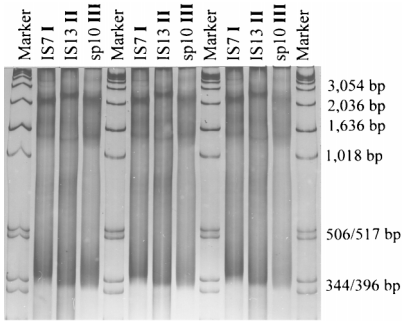

FIG. 2. Silver-stained SSCP gel showing the three *fla* alleles and the reproducibilities of the banding patterns among runs. The sample and allele designations (roman numerals) are indicated. Size markers consisted of a 1-kb ladder (Bethesda Research Laboratories).

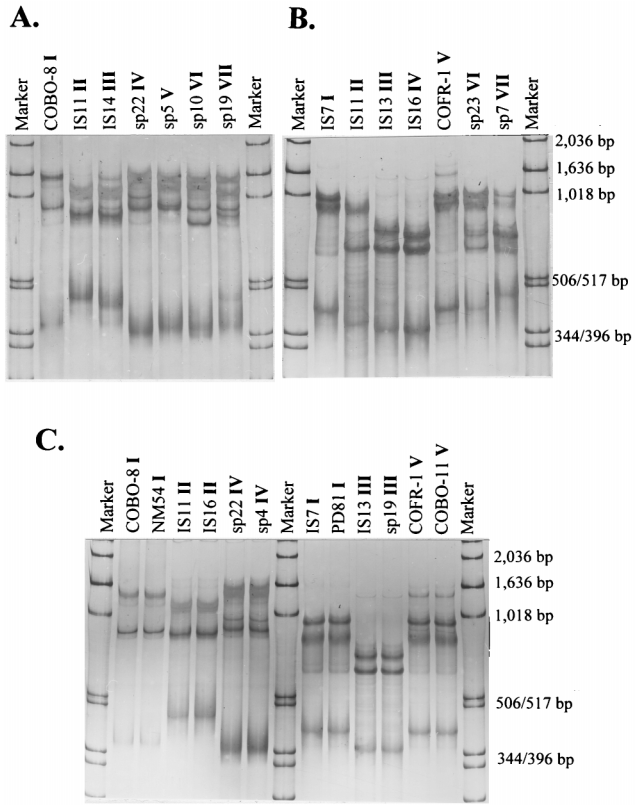

FIG. 3. (A) Silver-stained SSCP gel showing the seven *ospA* alleles. (B) Silver-stained SSCP gel showing the seven *p66* alleles. (C) Silver-stained SSCP gel showing the reproducibilities of the banding patterns of the *ospA* and *p66* alleles. Two representatives of three alleles for each gene were run. The sample and allele designation (roman numerals) are indicated. Size markers consisted of a 1-kb ladder from Bethesda Research Laboratories.

TABLE 2. Numbers of substitutions between pairs of sequences in the *fla*, *p66*, and *ospA* genes in *B. burgdorferi*

Gene and allele	No. of substitutions[a]												
	I	I	II	II	III	III	IV	IV	V	V	VI	VI	VII
fla													
I	0		11:0	11:0	10:0								
I			11:0	11:0	10:0								
II				0	1:0								
II					1:0								
p66													
I	0	0		9:2	11:3	11:3	11:3		2:1	2:1	2:2	2:2	11:2
I		0		9:2	11:3	11:3	11:3		2:1	2:1	2:2	2:2	11:2
I				9:2	11:3	11:3	11:3		2:1	2:1	2:2	2:2	11:2
II					2:1	2:1	4:3		11:3	11:3	11:4	11:4	14:2
III						0	4:2		13:4	13:4	13:5	13:5	13:5
III							4:2		13:4	13:4	13:5	13:5	13:5
IV									13:4	13:4	13:5	13:5	13:5
V										0	4:1	4:1	13:3
V											4:1	4:1	13:3
VI												0	13:4
VI													13:4
ospA													
I	0		8:10	8:10	7:10		7:9	7:9	3:3	3:3	5:3	5:3	4:3
I			8:10	8:10	7:10		7:9	7:9	3:3	3:3	5:3	5:3	4:3
II				0	3:4		3:3	3:3	7:9	7:9	6:7	6:7	7:9
II					3:4		3:3	3:3	7:9	7:9	6:7	6:7	7:9
III							2:3	2:3	6:7	6:7	8:7	8:7	8:7
IV								0	6:8	6:8	8:6	8:6	6:6
IV									6:8	6:8	8:6	8:6	6:6
V										0	4:2	4:2	3:2
V											4:2	4:2	3:2
VI												0	4:2
VI													4:2

[a] The number of substitutions appear as number of transitions: number of transversions. For example, there were nine transitions and two transversion between *p66* allele I and II. Comparison between alleles with identical SSCP profiles are underlined.

and each had identical sequences. In *p66*, four of the seven alleles were sequenced from different isolates and all had identical sequences, and in *ospA*, six of the seven alleles were sequenced and had identical sequences. For all alleles, sequences were different when SSCP patterns varied. The sensitivities and specificities of the SSCP analyses were therefore 100% and were as high as those in previous studies (7, 18, 29). Note that SSCP analysis detected differences as slight as a single substitution (e.g., a transition between *fla* allele II versus allele III; Table 2).

Allele frequencies and Shannon diversity indices at each of the three loci appear in Table 3. Statistics are reported for all samples and are then subdivided into those for cultured and uncultured *B. burgdorferi* isolates. Cultured *B. burgdorferi* organisms were isolated from triturated ticks and were grown and passed in BSK-H culture medium prior to being mixed with glycerol and frozen. Genes from uncultured *B. burgdorferi* isolates were amplified directly from DNA isolated from whole infected tick homogenates. Table 3 compares the allele frequencies from cultured and uncultured *B. burgdorferi* isolates collected at a single location in Larimer County, Colo. Allele frequencies were compared by Fisher's exact test. The frequencies of alleles in *p66* and *ospA* are significantly different between cultured and uncultured *B. burgdorferi* isolates. Differences in frequency and diversity were largest among alleles at the *p66* and *ospA* loci. There were 147 possible three-locus genotypes (three *fla* alleles · seven *p66* alleles · seven *ospA* alleles), of which we observed 16. One of these was observed in both ticks and cultures, six occurred only in cultured organisms, and nine were found only in ticks. In addition to having fewer genotypes, Shannon diversity indices are consistently lower for cultured *B. burgdorferi* isolates.

DISCUSSION

A great diversity of genotypes exists among the *B. burgdorferi* isolates circulating in the enzootic cycle in Colorado. A lower amount of diversity was observed in the *fla* locus, but this was expected given the sequence conservation of the *fla* gene (20, 42, 47) and the constraints on structure dictated by flagellar function. Our results demonstrate large and significant differences in the frequencies of alleles in cultured versus uncultured *B. burgdorferi* isolates and indicate that the diversity of haplotypes is greatly reduced in cultured spirochetes.

These results suggest that the process of culturing *B. burgdorferi* in BSK-H medium may isolate a limited number of the genotypes circulating in a population. There are at least three explanations for this phenomenon. First, established culturing methods and various aspects of the BSK-H medium (e.g., pH, molarity of salts, temperature, and antibiotics) may allow for the survival of only a few genotypes. Second, culturing methods and the BSK-H medium may allow some genotypes to outcompete other genotypes in culture (19, 40, 41). Third, the decline in diversity and shifts in allele frequencies could arise during severe bottlenecks in the survival of *B. burgdorferi* genotypes during the culturing process. However, bottlenecks would remove genotypes at random and fail to explain why particular alleles (e.g., *p66* allele I and *ospA* allele I) arise

TABLE 3. Haplotype frequency among 71 Colorado *B. burgdorferi* samples partitioned by source

Gene and allele	No. (%) of samples			P^c	No. (%) of samples		P
	All samples	All sites			Larimer County		
		Culture[a]	Tick[b]		Culture	Tick	
fla (three haplotypes; 390 bp)							
I	65 (0.916)	43 (0.915)	22 (0.916)	0.557	32 (0.889)	20 (0.909)	0.333
II	5 (0.070)	4 (0.085)	1 (0.042)		4 (0.111)	1 (0.045)	
III	1 (0.014)	0 (0.000)	1 (0.042)		0 (0.000)	1 (0.045)	
Diversity[d]	0.471	0.420	0.500		0.503	0.528	
p66 (seven haplotypes; 236 bp)							
I	42 (0.592)	40 (0.851)	2 (0.083)	5×10^{-14}	32 (0.889)	2 (0.091)	1.2×10^{-11}
II	1 (0.014)	1 (0.021)	0 (0.000)		1 (0.028)	0 (0.000)	
III	5 (0.070)	2 (0.043)	3 (0.125)		2 (0.056)	3 (0.136)	
IV	1 (0.014)	1 (0.021)	0 (0.000)		1 (0.028)	0 (0.000)	
V	3 (0.042)	3 (0.064)	0 (0.000)		0 (0.000)	0 (0.000)	
VI	18 (0.254)	0 (0.000)	18 (0.750)		0 (0.000)	16 (0.727)	
VII	1 (0.014)	0 (0.000)	1 (0.042)		0 (0.000)	1 (0.045)	
Diversity	1.669	0.881	1.176		0.673	1.242	
ospA (seven haplotypes; 345 bp)							
I	57 (0.803)	42 (0.894)	15 (0.625)	1×10^{-4}	31 (0.861)	13 (0.591)	2.6×10^{-4}
II	4 (0.056)	4 (0.085)	0 (0.000)		4 (0.111)	0 (0.000)	
III	1 (0.014)	1 (0.021)	0 (0.000)		1 (0.028)	0 (0.000)	
IV	3 (0.042)	0 (0.000)	3 (0.125)		0 (0.000)	3 (0.136)	
V	2 (0.028)	0 (0.000)	2 (0.083)		0 (0.000)	2 (0.091)	
VI	3 (0.042)	0 (0.000)	3 (0.125)		0 (0.000)	3 (0.136)	
VII	1 (0.014)	0 (0.000)	1 (0.042)		0 (0.000)	1 (0.045)	
Diversity	1.188	0.564	1.664		0.682	1.747	

[a] Culture, sample originating from spirochete culture ($n = 47$).
[b] Tick, sample originating from tick extracted DNA ($n = 24$).
[c] Fisher's exact test.
[d] Shannon's diversity index.

consistently more often in cultured isolates. We therefore suggest that selection of particular genotypes occurs during the culturing process. We have no basis for suggesting that the *p66* allele I or the *ospA* allele I directly confer a greater ability to survive the process of culturing.

Similar patterns have appeared in earlier studies that involved culturing of *B. burgdorferi* from field collections of ticks or vertebrate hosts. The frequencies of *B. burgdorferi* clones with high- and low-infectivity phenotypes were compared after several in vitro passages in BSK medium, and it was found that low-infectivity clones are lost from culture during the first five passages (31). Our results suggest that isolates may be selected against even more rapidly, perhaps in the initial culture. *B. burgdorferi* could be cultured in BSK II medium from only 7 to 10% of *Ixodes ricinus* ticks in the United Kingdom that were PCR positive with *ospA* primers (25). Likewise, *B. burgdorferi* sensu lato was cultured in BSK II medium from only 23% of *I. ricinus* ticks from Valais, Switzerland, that were positive by indirect immunofluorescence detection (34). A low culture rate in BSK II medium was reported in *I. ricinus* ticks from Giessen, Germany (49), that were PCR positive with *fla* primers. That study also demonstrated that additives to BSK II medium increase the culture rate. *Borrelia lonestari*, the possible agent of a Lyme disease-like illness, is uncultivatable (3).

The observation of alleles distributed exclusively among uncultured spirochetes is significant because it suggests that in the *B. burgdorferi* population circulating between *I. spinipalpis* and wood rat populations in Colorado, a greater diversity of genotypes exist than is detected through the current culturing process. We strongly suggest that this work be repeated in regions of the United States where human Lyme disease is highly endemic and where underestimation of genetic diversity might affect the development of prophylactics and diagnostic tools. It will be interesting to compare the diversity and numbers of alleles detected among cultured isolates in Colorado with those cultured from humans, mice, and ticks in the Northeast, upper Midwest, Southeast, and California. The appearance of the same genotypes in cultures from these regions would provide strong evidence for a common mechanism of selection exerted on a diversity of *B. burgdorferi* genotypes by culturing in BSK medium.

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